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First record of *Morchella pulchella* from Pakistan

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Abstract—A species of true morel (*Morchella*) was collected in the Malam Jabba valley in the Swat District of Pakistan in April 2015. The specimen was identified by sequencing portions of RNA polymerase II largest subunit (RPB1), second largest subunit (RPB2), and translation elongation factor-1α (tef1). Phylogenetic analysis of the partial tef1 sequence indicated the collection was *M. pulchella*, previously reported from China, Turkey, and Europe. Our report extends its distribution range to Pakistan.

Key words—*Ascomycota*, edible fungi, *Morchellaceae*, phylogeny, systematics

Introduction

Malam Jabba is located in northern Pakistan between the Himalayan and Hindukush foothills in the Swat district of the Malakand Division in Khyber Pakhtunkhwa Province (KP). The area, with elevations ranging from 990 m at the valley entrance to 2880 m at the highest peak of Shagar Sar, is a mycologically rich region. In Pakistan, *Morchella* species fruit during spring at lower altitudes, but as late as July at higher elevations in snowfall regions (Hamayun & al. 2006, Badshah & al. 2015). True morels (*Morchella* spp.) belong to *Morchellaceae* Rchb. (*Pezizales*, *Ascomycota*) and are amongst the most highly valued edible fungi due to their excellent flavor and relatively short fruiting season (Taşkın & al. 2016). A high diversity of *Morchella* species has been described from various continents and regions, including Pakistan (Hamayun & al. 2006).

In recent years, a series of multilocus molecular phylogenetic studies of *Morchella* were performed employing phylogenetic species recognition based on genealogical concordance (GCPSR, sensu Taylor & al. 2000). These studies revealed considerable continental endemism and provincialism within the genus (O'Donnell & al. 2011; Du & al. 2012a,b; Richard & al. 2015; Loizides & al. 2016; Taşkın & al. 2010, 2012, 2016). This research also highlighted the difficulties surrounding morphology-based species identification and helped provide a robust framework for subsequent taxonomic revisions (Clowez 2012, 2014, 2015; Kuo & al. 2012; Richard & al. 2015; Voitk & al. 2016; Loizides & al. 2015, 2016). Multilocus molecular phylogenetic studies have so far revealed nearly 70 *Morchella* spp. worldwide (Loizides 2017).

A number of morel species—*Morchella conica, M. crassipes*, *M. elata, M. esculenta, M. rotunda, M. semilibera*—have been previously reported from the Kohistan and Swat regions of Pakistan based solely on morphology (Hamayun & al. 2006). These identifications based on old taxonomical concepts have not, however, been verified by molecular analysis. In this paper we report *Morchella pulchella* as a new record from Pakistan identified primarily using DNA sequence analysis.

Materials & methods

Specimens were collected during a field survey at Malam Jabba, District Swat, Province Khyber Pakhtunkhwa, Pakistan, in April 2015 under *Pinus wallichiana* near grasses. Tissues were mounted in glycerin or distilled water and examined microscopically using a Leitz HM Lux compound microscope under 400× magnification. The preserved specimens have been deposited in the herbarium of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan (ISL).

DNA extraction and PCR amplification were conducted with the Sigma-Aldrich REDExtract-N-Amp™ Plant PCR Kit following the manufacturer's instructions. DNA was amplified with the following markers and primer pairs: translation elongation factor 1-α (TEF1) with EF526F & EF3AR (Rehner & Buckley 2005), RNA polymerase II large subunit (RPB1) with gRPB1A & aRPB1C (Matheny & al. 2002) and the RNA polymerase II second largest subunit (RPB2) with 9F & 3R (Liu & al. 1999). One μL of genomic DNA was amplified in total volumes of 20 μL in an Eppendorf Master Cycler Gradient thermocycler following the parameters of 94°C for 3 min, 35 cycles of 94°C for 30 s, 53°C for 30 s, 72°C for 1–2 min with a final

FIG. 1—Phylogenetic tree inferred from partial TEF1 sequences of *Morchella* spp., using the Neighbor Joining method. Posterior probabilities and bootstrap values are given on each node of the tree; the values for poorly supported nodes (bootstrap <40) are enclosed in parentheses. The new Pakistani sequence is set in bold font.

M. sp. HM056335
M. sp. JN085100
M. fekeenis JN085077
0.99/100
M. fekeenis JN085074
M. fekeenis JN085058
M. brunnea GU551397
0.88/71
M. brunnea GU551385
M. brunnea GU551378
M. brunnea GU551377
M. pulchella KM491180
M. septentrionalis KM491186
M. septentrionalis KM491187
0.96/45
M. septentrionalis JN084942
(0.93/34)
M. pulchella HM056344
M. pulchella JQ321857
M. pulchella JN085057
M. pulchella HM056341
(0.74/19)
M. pulchella MF400857
M. pulchella JN085068
M. sp. GU551544
M. sp. GU551381
(0.75/30)
M. sp. GU551390
M. conifericola JN085084
M. conifericola JN085071
0.95/68
0.005
0.97/62
M. conifericola JN085069
0.99/68
M. conifericola JN085060

extension at 72°C for 7 min. Raw sequence data were edited using BioEdit (Hall 1999), assembled with Codon Code Aligner 4.1.1, and deposited in GenBank.

Sequences were analyzed using the BLASTn analysis tool (www.ncbi.nlm.nih.gov). Our newly generated TEF1 sequence plus 26 TEF1 sequences representing *Morchella* species previously deposited in NCBI were used for the phylogenetic analysis. DNA sequences were aligned using Muscle E multiple alignment tool within MEGA v. 7.0 (Kumar & al. 2016). A phylogenetic tree was inferred using the Neighbour-Joining method. Posterior probabilities were determined and the bootstrap analysis was based on 1000 pseudo-replicates using the NJ option.

Results

BLASTn analysis using the TEF1 sequence (FIG. 1) as query matched three GenBank sequences representing *M. pulchella* (KM491180: 100% identity, 636/636 bp) and an unidentified *Morchella* sp. (GU551381: 100% identity, 636/636 bp; GU551390: 99% identity, 635/636 bp).

Similarly, the RPB1 sequence matched three GenBank sequences representing *M. septentrionalis* (KM588048 & JX173414: 100% identity, 785/785 bp) and *M. pulchella* (HM056439: 100% identity, 785/785 bp).

Finally, the RPB2 sequence matched three GenBank sequences representing *M. pulchella* (KM588041, 100% identity, 809/809 bp) and *M. septentrionalis* (KM588048 & JX173414: 100% identity, 805/805 bp).

Taxonomy

Morchella pulchella Clowez & Franç. Petit,
Bull. Soc. Mycol. Fr. 126: 314. 2012. FIG. 2

ASCOMATA 60–70 × 52–56 mm, conical, brown. HYMENOPHORE 50 × 40 mm, slightly conical, ridged with 26 primary ridges per ascoma, ridges regular to irregular in shape, 2–5 mm long, 3 mm broad, pitted, pits (n = 55–60) short and elongated. Fertile area sharply attached to the stipe. STIPE hollow, white, convex, tapered at apex and wider at base with short rays. ASCOSPORES 20.4–24 × 13–15.6 µm, ellipsoid, smooth, with homogenous contents. ASCI 8-spored, cylindrical, hyaline, 200–260 × 15–20 µm, length/width (Qm) ratio 1.5 µm. PARAPHYSES shorter than asci, 78–150 × 10–14 µm, cylindrical, subclavate, apices rounded, narrow, mostly subacute, rarely acute, septate, hyaline with homogenous contents; yellow hyphal elements on sterile ridges 77–181 × 7.9–26 µm.

MATERIAL EXAMINED—**PAKISTAN, KHYBER PAKHTUNKHWA, Swat District**, Malam Jabba, 34°47′54″N 72°34′33″E, altitude 1800 m, under *Pinus wallichiana* A.B. Jacks. and local grasses, 15 April 2015, coll. Hussain Badshah B10 (ISL-67968; GenBank MF400855, MF400856, MF400857).

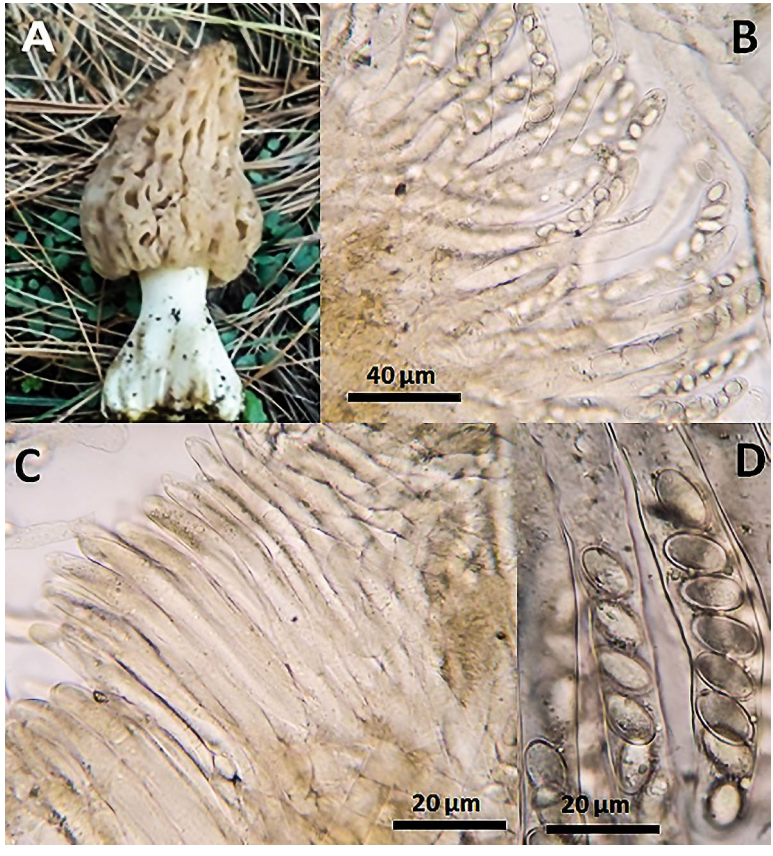

Fig. 2—*Morchella pulchella* (ISL-67968)
A: ascocarp. B, D: asci with ascospores. C: paraphyses.

Discussion

The phylogenetic analysis (Fig. 1) clustered sequences from our collection with *M. pulchella*, supporting the Malam Jabba specimen as a new record of that species from Pakistan. As in previous studies (Du & al. 2012, Taşkın & al. 2012, Richard & al. 2015), our phylogenetic analysis provides no support for the reciprocal monophyly of *M. pulchella* and *M. septentrionalis* and emphasizes the need to investigate further the taxonomic status of *M. pulchella* and *M. septentrionalis*.

Morchella pulchella has previously been reported only from China (Du & al. 2012a,b), Turkey (Taşkin & al. 2010), France (Clowez 2012, Richard

& al. 2015), and Spain (Alonso & al. 2016). Confirmation of *M. pulchella* from an apparently undisturbed mountainous site in Pakistan suggests that *M. pulchella* is unlikely to have been introduced to this region through human activity and has an innately Eurasian distribution.

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